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Identification of Cocarcinogens and Their Potential Mechanisms of Action Using C3H10T(1/2)CL8 Mouse Embryo Fibroblasts

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Identification of Cocarcinogens and Their Potential Mechanisms of Action Using C3H10T½CL8 Mouse Embryo Fibroblasts[1]

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ABSTRACT

The cocarcinogenic action of five agents which increase microsomal mixed-function oxidase activity *in vivo* was examined in the C3H10T½CL8 transformation assay. The compounds studied were benz(*a*)anthracene, 5,6-benzoflavone, phenobarbital, pregnenolone-16α-carbonitrile, and Aroclor 1254. After a 48-hr pretreatment with the agent, the cells were then treated with benzo(*a*)pyrene [B(a)P] and the agent for an additional 24 hr. All agents except for Aroclor 1254 increased B(a)P-mediated transformation in C3H10T½CL8 cells. Benz(*a*)anthracene, 5,6-benzoflavone, phenobarbital, and pregnenolone-16α-carbonitrile also increased the overall metabolism of B(a)P in C3H10T½CL8 cells to 9,10-dihydro-9,10-dihydroxybenzo(*a*)pyrene; 7,8-dihydro-7,8-dihydroxybenzo(*a*)pyrene, 9-hydroxybenzo(*a*)pyrene, and 3-hydroxybenzo(*a*)pyrene. Growth studies indicated that all four agents had no stimulatory effect which might have explained the increases in transformation frequency. This suggests that these agents exert their cocarcinogenic action via increases in the enzyme-mediated pathways of B(a)P metabolism.

INTRODUCTION

The C3H10T½CL8 mouse embryo fibroblast cell line is particularly valuable as an *in vitro* assay for detecting carcinogens, cocarcinogens (20), and tumor promoters (13, 17, 18) as well as for studying the basic mechanisms of carcinogenesis. This cell line is highly sensitive to postconfluence inhibition of cell division, has a very low incidence of spontaneous transformation, and can be transformed by X-rays, polycyclic aromatic hydrocarbons, alkylating agents, cigarette smoke condensate, hair dyes, and some antineoplastic agents (2–5, 13, 22, 28). C3H10T½CL8 cells contain the mixed-function oxidase, epoxide hydrase, and conjugating enzymes that are capable of metabolizing carcinogens, such as B(a)P[3] (9) and 3-methylcholanthrene (9, 20). These enzymes are responsible for both the formation and detoxification of carcinogenic intermediates derived from the parent chemical carcinogen.

In mouse liver, phenobarbital, 5,6-benzoflavone, and pregnenolone-16α-carbonitrile induce formation of different cytochromes which exhibit different enzymatic and electrophoretic properties (11). Aroclor 1254 also induces aryl hydrocarbon hydroxylase in mouse liver (12), while benz(*a*)anthracene has been shown to induce B(a)P-metabolizing enzymes in mouse skin (10). In C3H10T½CL8 cells, benz(*a*)anthracene induces a microsomal cytochrome P-448 and induces microsomal enzyme activity as measured by increased metabolism of 3-methylcholanthrene (10, 18). Since these agents affect the enzymes which activate and detoxify chemical carcinogens, they might affect the transformation response elicited by carcinogens. This phenomenon has been reported by Kouri *et al.* (14), who found that 2,3,7,8-tetrachlorodibenzo-*p*-dioxin exerted a cocarcinogenic action on 3-methylcholanthrene-treated mice. This study was performed to examine the potential cocarcinogenic effects of phenobarbital, benz(*a*)anthracene, 5,6-benzoflavone, pregnenolone-16α-carbonitrile, and Aroclor 1254 on B(a)P-induced transformation of C3H10T½CL8 mouse embryo fibroblasts. Their potential mechanism of action was investigated by studying their effect on the metabolism of B(a)P and on the growth characteristics of C3H10T½CL8 cells.

MATERIALS AND METHODS

Chemicals and Radioactive Compounds. [G-^{3}H]B(a)P (24 to 40 Ci/mmol) was purchased from Amersham, Arlington Heights, Ill. Purification of [^{3}H]B(a)P was accomplished by high-pressure liquid chromatography using a Dupont Zorbax-ODS column (4.6 mm inside diameter x 24 cm) (Dupont Instruments, Wilmington, Del.) and 100% methanol as the elution solvent. The retention time of B(a)P at 2000 psi was 6 min. Nonradiolabeled B(a)P was added to the purified radiolabeled B(a)P to adjust the specific activity to 340 to 380 μCi/μmol. The B(a)P and benz(*a*)anthracene were purchased from Sigma Chemical Co., St. Louis, Mo. Spectrograde acetone and 5,6-benzoflavone were purchased from Aldrich Chemicals, Milwaukee, Wis. Spectrograde methanol, ethyl ether, and ethyl acetate were purchased from Burdick and Jackson, Muskegon, Mich. Phenobarbital sodium was purchased from Mallinckrodt Chemical Co., St. Louis, Mo.

Cells and Culture Conditions. The mouse embryo fibroblast cell line C3H10T½CL8, as derived by Reznikoff *et al.* (22, 23) and kindly donated by Dr. Charles Heidelberger, was used in these experiments. Cell cultures were incubated in humidified incubators (Forma Scientific, Marietta, Ohio, or Hotpack, Philadelphia, Pa.) with an atmosphere of 5% CO_2 in air at 37°. All cultures were grown in Eagle's basal medium with Earle's salts and L-glutamine supplemented with 10% heat-inactivated fetal

[1] A preliminary account of this work was presented at the 70th Annual Meeting of the American Association for Cancer Research in New Orleans, La., May 16 to 19, 1979 (21).

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[3] The abbreviations used are: B(a)P, benzo(*a*)pyrene; B(a)P-pre-9,10-diol, all radioactivity eluted prior to 9,10-diol; B(a)P-9,10-diol, 9,10-dihydro-9,10-dihydroxybenzo(*a*)pyrene; B(a)P-4,5-diol, 4,5-dihydro-4,5-dihydroxybenzo(*a*)pyrene; B(a)P-7,8-diol, 7,8-dihydro-7,8-dihydroxybenzo(*a*)pyrene; B(a)P-1,6-quinone, benzo(*a*)pyrene 1,6-quinone; B(a)P-6,12-quinone, benzo(*a*)pyrene 6,12-quinone; B(a)P-9-phenol, 9-hydroxybenzo(*a*)pyrene; B(a)P-3-phenol, 3-hydroxybenzo(*a*)pyrene; B(a)P-3,6-quinone, benzo(*a*)pyrene 3,6-quinone; B(a)P-7,8-oxide, benzo(*a*)pyrene 7,8-oxide; B(a)P-*trans*-7,8-diol, *trans*-7,8-dihydro-7,8-dihydroxybenzo(*a*)pyrene.

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calf serum (Grand Island Biological Co., Grand Island, N. Y.). The cells were routinely checked for *Mycoplasma* contamination and found to be *Mycoplasma* free.

Oncogenic Transformation Assay. Cells were seeded onto 60-mm Petri dishes (Falcon, Cockeysville, Md.) (1000/dish) in 5 ml of medium (12 dishes/experimental point) and were treated 24 hr later with the test agent. Forty-eight hr later, the cultures were refed and exposed to 4 μM B(a)P and the test agent for 24 hr. The medium was removed, and the cells received fresh complete medium containing penicillin (100 units/ml) and streptomycin (50 μg/ml) (Grand Island Biological Co.). Media were changed weekly until the cells reached confluency, whereupon the fetal calf serum concentration was reduced to 5%.

At the end of 6 weeks, the dishes were washed with 0.85% NaCl solution, fixed with methanol, stained with Giemsa, and scored for oncogenic transformation. Three different types of foci have been described after treatment of C3H10T½CL8 cells with polycyclic aromatic hydrocarbons. Only type II and type III foci were scored, since it has been demonstrated that these foci produce sarcomas upon injection into irradiated C3H mice (22, 23).

Control cultures received the appropriate solvent and were treated in the same manner as were the exposed cells. Cytotoxicity assays were run concurrently with the transformation assays and were performed using the same protocol, except that the dishes were plated with 200 cells (6 dishes/experimental point) and that the dishes were stained 10 to 12 days after plating.

High-Pressure Liquid Chromatographic Analysis of Metabolites. Cells were seeded into 25-sq cm flasks (Falcon) at 25,000 to 50,000 cells/flask with 3 flasks/experimental point and each point performed in duplicate. The cells were exposed to the test agent for 48 hr. The concentration of test agent used in the metabolite study was the one that exerted a maximal transformation response in the oncogenic transformation assay. The cultures were refed with fresh complete medium and treated with [^{3}H]B(a)P (4 μM, 4 to 5 μCi/flask) and the test agent for 24 hr. At the end of this exposure time, the medium was removed, and the cells were trypsinized and lysed by freeze-thawing. The cells and media were combined and extracted with ethyl acetate:acetone (2:1) and centrifuged for 10 min at 1100 × *g*. An aliquot consisting of 80% of the organic layer was removed, evaporated under dry nitrogen, and stored at −80°.

High-pressure liquid chromatography was performed on a Dupont Model 848 chromatograph fitted with a 4-mm (inside diameter) x 30-cm C_{18}-μBondapak column (Waters Associates, Milford, Mass.). The samples were reconstituted in methanol: acetone (1:2), cold B(a)P metabolite standards were added, and the mixture was injected into the chromatograph. The eluting solvent consisted of 66.3% methanol, 30.4% deionized water, and 3.3% ethyl ether run isocratically at ambient temperature and at a flow rate of 1.4 ml/min. The eluant was monitored by UV at 254 nm, and eluted fractions were collected for liquid scintillation counting (27). The radiochemical data were quantitated by computer analysis. The overall recovery of radioactivity in these experiments was greater than 90%. The percentage of B(a)P metabolized to organic soluble metabolites was between 1 and 8%, and the percentage metabolized to aqueous soluble metabolites was between 1 and 4% of the total radioactivity applied. Controls for the air oxidation of B(a)P consisted of flasks with media and [^{3}H]B(a)P and were subjected to the same experimental conditions described above. Each data point presented has been adjusted for this activity.

Effect on Growth of C3H10T½CL8 Cells. Cells were seeded into 60-mm Petri dishes at 5000/dish (5 dishes/point); 96 hr after seeding, the cells were refed with complete medium, treated with the appropriate agent which remained until harvest, and counted daily using a Model ZBI Coulter Counter (Coulter Electronics, Hialeah, Fla.).

RESULTS

Oncogenic Transformation in Culture. The effect of the various agents on B(a)P-induced oncogenic transformation of cells in culture was investigated using C3H10T½CL8 cells (Table 1). Cells treated only with the modifier produced no transformation, whereas cells treated with B(a)P gave a maximum transformation frequency of 6.75 to 16.3 type II and III foci/10^3 survivors at 4 μM and a survival of 60 to 80%.

Pretreatment of C3H10T½CL8 cells with benz(*a*)anthracene at 1 μM for 48 hr gave a 2.4-fold increase in B(a)P-mediated transformation with no increase in B(a)P-mediated cytotoxicity. Similarly, 5,6-benzoflavone produced a 1.6-fold increase in transformation compared to those cultures treated with B(a)P

Table 1

Cocarcinogenic effect of various agents on B(a)P-mediated transformation in C3H10T½CL8 cells

Cells were seeded at 1000/dish; 24 hr after plating, the cells were treated with the agent. Forty-eight hr later, the cells were treated again with the agent and B(a)P (4 μM). Twenty-four hr later, the chemicals were removed. The percentage of survival of cells treated with B(a)P (4 μM) alone was 60 to 80%, with a plating efficiency of 15 to 20%. At all concentrations tested, cells treated with the agent and B(a)P showed no change in cytotoxicity except with the pregnenolone-16α-carbonitrile experiment whose values were the plating efficiencies (percentage), acetone control, 21; B(a)P control, 15; pregnenolone-16α-carbonitrile and B(a)P:0.3 μM, 15; 1.0 μM, 8.5; 3.0 μM, 7.6; 10 μM, 7.3; and 30 μM, 6.5.

	Total type II and III foci/10^3 survivors			Transformation with B(a)P and agent [total type II and III foci/10^3 survivors]					
Agent	Acetone control	Agent control[a]	B(a)P control (no agent)	0.3 μM agent	1.0 μM agent	3.0 μM agent	10.0 μM agent	30.0 μM agent	100.0 μM agent
Benz(*a*)anthracene	<0.380[b]	<0.424[b]	6.75	8.1 (1.2)[c]	16.2 (2.4)	11.5 (1.7)	4.73 (0.7)	4.05 (0.6)	NT[d]
5,6-Benzoflavone	<0.329[b]	<0.298[b]	7.98	7.26 (0.91)	8.78 (1.1)	11.1 (1.4)	12.8 (1.6)	6.07 (0.76)	NT
Phenobarbital	<0.365[b]	<0.316[b]	12.9	12.1 (0.93)	14.3 (1.1)	12.9 (1.0)	12.9 (1.0)	25.9 (2.0)	23.4 (1.8)
Pregnenolone-16α-carbonitrile	<0.390[b]	<0.595[b]	9.84	7.48 (0.76)	12.7 (1.3)	16.7 (1.7)	17.7 (1.8)	13.9 (1.42)	ND
Aroclor 1254	<0.349[b]	<0.349[b]	16.3	18.6 (1.1)	22.6 (1.4)	15.8 (0.97)	17.5 (1.07)	16.3 (1.00)	NT

[a] Dishes treated only with agent at 10 μM. Dishes treated only with agent at other concentrations produced no type II or type II foci.
[b] No type II or III foci were observed in any of these controls.
[c] Numbers in parentheses, ratio of B(a)P and agent group to B(a)P control group.
[d] NT, not tested; ND, not detected.

only, again with no increase in B(a)P-mediated cytotoxicity. Phenobarbital (30 μM) and pregnenolone-16α-carbonitrile (10 μM) gave a 2- and 1.8-fold increase in transformation, respectively. The increases in transformation elicited by benz(*a*)-anthracene, 5,6-benzoflavone, and phenobarbital were a result of an absolute increase in the number of foci per dish, while that seen with pregnenolone-16α-carbonitrile was a result of an increase in overall cytotoxicity caused by the test agent. The polychlorinated biphenyl Aroclor 1254 gave a marginal increase in B(a)P-mediated transformation of 40% at 1 μM. The results presented in Table 1 represent the data from single experiments. However, each experiment was repeated several times with similar results.

Analysis of B(a)P Metabolites. High-pressure liquid chromatography was utilized in order to examine the effect of these modifiers upon B(a)P metabolism in cultures of C3H10T½CL8 cells. Log-phase cultures of C3H10T½CL8 cells, pretreated for 48 hr with modifier, were incubated with [^{3}H]B(a)P for 24 hr, and organic-soluble metabolites were isolated and separated by high-pressure liquid chromatography (27). The following metabolites were analyzed for: B(a)P-pre-9,10-, 9,10-, 4,5-, and 7,8-dihydrodiols; B(a)P-1,6-, 3,6-, 6,12-quinones; and B(a)P-9- and 3-phenols.

A chromatogram of the B(a)P metabolites formed by C3H10T½CL8 cells in culture after a 24-hr exposure to B(a)P is illustrated in Chart 1. The chromatogram revealed substantial amounts of B(a)P-9,10- and 7,8-diols and smaller quantities of B(a)P-9-phenol, B(a)P-3-phenol, and B(a)P-3,6-quinone. B(a)P-1,6-quinone, B(a)P-6,12-quinone, and B(a)P-4,5-diol were not formed to any extent.

Table 2 compares the absolute amounts of organic soluble B(a)P metabolites produced by C3H10T½CL8 cells treated with [^{3}H]B(a)P to cells treated with the agent and [^{3}H]B(a)P. The treated cells were preexposed to the agent for 48 hr and then to [^{3}H]B(a)P and the agent for an additional 24 hr. Forty-eight hr was chosen as the preincubation period based on previous experiments on microsomal enzyme induction (20). The agent was added to the cells with [^{3}H]B(a)P in order to prevent loss of induced enzyme activity. It has been determined in hamster embryo cells that the half-life of mixed-function oxidase is 3 hr (19). The concentration of the test agent added was that which gave the maximal transformation response. As seen in Tables 1 and 2, both the transformation frequencies and metabolic rates of cells treated only with B(a)P varied from experiment to experiment. This variation is probably a result of differences in fetal calf serum, media, plasticware, and age of the cells. Benz(*a*)anthracene produced a 1.9-fold increase in total B(a)P metabolite formation, with an increase in total B(a)P-7,8-diol formation of 1.8-fold. For 5,6-benzoflavone, the overall increase in metabolite formation in C3H10T½CL8 cells was 2-fold. The individual metabolite formation between untreated and treated cells also showed approximately a 2-fold increase for each metabolite, except for those metabolites with levels that are near the detection limits of the system. Phenobarbital

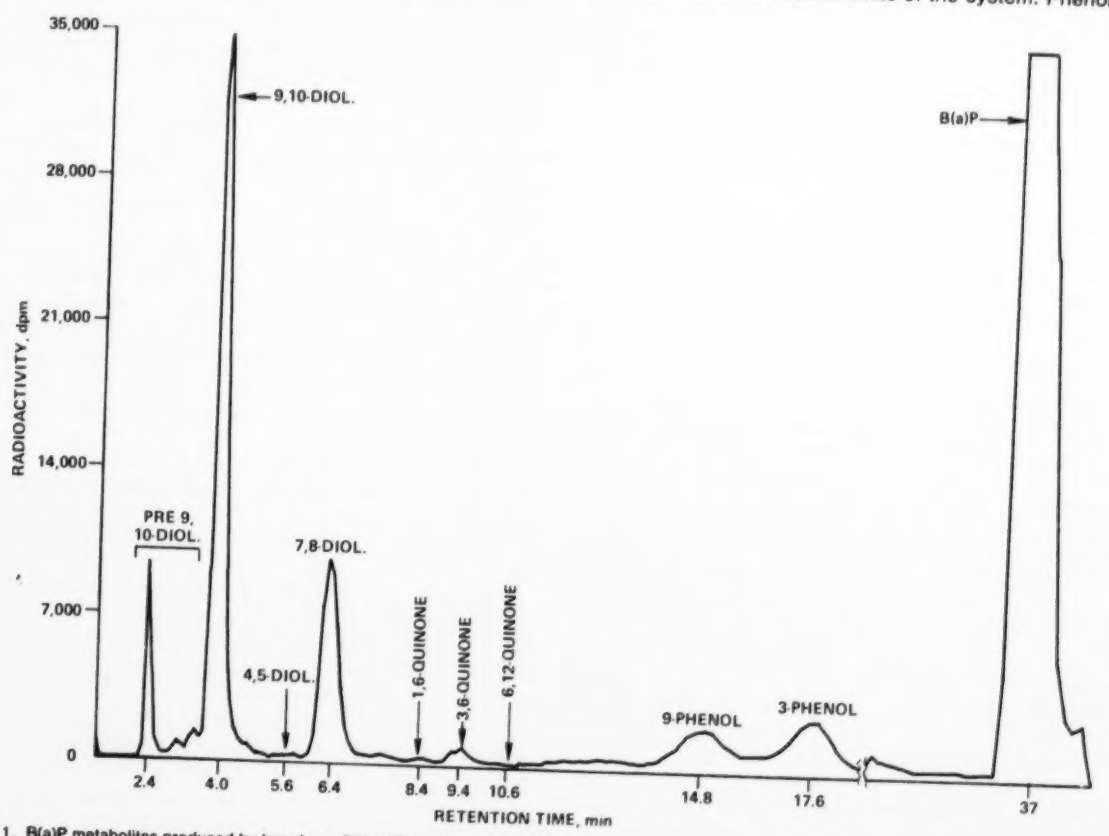

Chart 1. B(a)P metabolites produced by log-phase C3H10T½CL8 mouse embryo fibroblasts in culture after 24 hr. See "Materials and Methods" for details.

Tab

B(a)P metabolites formed

Cells were seeded at 25,000 to 50,000 per flask; 24 hr after plating, the cells were treated with the agent. The concentration of test agent applied to the cells was μM; Aroclor 1254, 3 μM. Forty-eight hr later, the cells were treated again with the agent and [^{3}H]B(a)P (4 μM, 340 to 380 μCi/μmol). Twenty-four hr later, the cells

				pmol B(a)P
	Pre-9,10-diol	9,10-Diol	4,5-Diol	7,8-Diol
B(a)P	16.4 ± 3.9[a] (7.9)[b]	109.5 ± 20.6 (52.6)	1.1 ± 0.8 (0.6)	49.9 ± 17.7 (23.4)
Benz(*a*)anthracene + B(a)P	37.9 ± 0.2 (9.8)	188.8 ± 7.3 (48.7)	3.9 ± 1.1 (1.0)	88.4 ± 7.0 (22.8)
B(a)P	31.0 ± 2.8 (9.4)	187 ± 1.0 (61)	1.9 ± 0.4 (0.5)	69.8 ± 2.3 (22.2)
5,6-Benzoflavone + B(a)P	72.4 ± 0.4 (11.1)	365 ± 13 (57.2)	3.5 ± 0.3 (0.5)	142 ± 9 (22.6)
B(a)P	28.3 ± 9.4 (7.2)	198.9 ± 38.1 (46.8)	3.4 ± 0.9 (0.8)	94.5 ± 25.2 (23.3)
Phenobarbital + B(a)P	57.2 ± 6.4 (8.6)	324.2 ± 58.0 (49)	7.2 ± 0.1 (1.0)	170.3 ± 14.5 (25.2)
B(a)P	18.1 ± 3.1 (10.1)	95.2 ± 4.9 (45.8)	2.9 ± 0.5 (1.6)	46.3 ± 2.9 (22.1)
Pregnenolone-16α-carbonitrile + B(a)P	21.3 ± 2.4 (7.1)	140 ± 8.0 (49.1)	2.7 ± 0.1 (1.0)	64.3 ± 2.0 (22.9)
B(a)P	37.1 ± 0.9 (12.9)	160.4 ± 2.4 (54.2)	3.2 ± 0.6 (1.2)	65.7 ± 2.5 (21.8)
Aroclor 1254 + B(a)P	27.7 ± 2.3 (11.1)	135.9 ± 6.6 (55.6)	2.0 ± 0.2 (0.8)	55.5 ± 4.3 (22.2)

[a] Mean ± range of duplicate determinations.
[b] Numbers in parentheses, percentage of total.

increased total B(a)P metabolite formation 1.7-fold. Pregnenolone-16α-carbonitrile increased B(a)P metabolism 1.4-fold. Data from numerous experiments with Aroclor 1254 showed that this agent produced either a slight inhibition or had no effect on B(a)P metabolite formation. In the data reported in Table 2, Aroclor 1254 exerted a slight inhibitory effect in overall metabolite formation.

Effect on Growth of C3H10T½CL8 Cells. A study was undertaken to investigate the effect of these agents on the growth of C3H10T½CL8 cells. Ninety-six hr after seeding, the cells were refed and treated with either the chemical or the appropriate solvent. The concentration of agent was that which gave maximal transformation response. A group of solvent-treated dishes was counted in order to provide comparative cell numbers.

A summary of the effect that 3 agents, benz(*a*)anthracene, 5,6-benzoflavone, and Aroclor 1254, have on the growth rate of C3H10T½CL8 cells is found in Table 3. Benz(*a*)anthracene, Aroclor 1254, and 5,6-benzoflavone inhibited the rate of cell division at the concentrations tested to different extents. This is in contrast to their lack of lethal toxicity to C3H10T½CL8 cells as determined by plating efficiency (Table 1). Similar experiments were performed with phenobarbital and pregnenolone-16α-carbonitrile. Neither agent enhanced or inhibited the rate of cell growth of C3H10T½CL8 cells.

DISCUSSION

B(a)P is metabolized via multiple pathways that are mediated by the mixed-function microsomal oxidases, epoxide hydrase, glutathione *S*-epoxide transferase, and UDP-glucuronic acid transferase. Many studies in a variety of cells (6, 26), organs (1, 25), and cell-free systems (9, 15) have demonstrated that a major pathway of metabolic activation of B(a)P occurs via mixed-function oxidase epoxidation at the 7,8 bond. Subsequent hydration of B(a)P-7,8-oxide to B(a)P-*trans*-7,8-diol by epoxide hydrase and further oxidation at the 9,10 double bond gives 7,8-dihydro-7,8-dihydroxybenzo(*a*)pyrene-9,10-oxide (24, 26). This activated electrophilic form of B(a)P binds to DNA and, in particular, is the major bound adduct to the DNA of C3H10T½CL8 cells treated with B(a)P (6).

The C3H10T½CL8 cells are capable of metabolizing certain carcinogens to their proximate and/or ultimate forms. Mixed-function oxidases (9, 20), epoxide hydrase (20), and NADPH-cytochrome *c* reductase (9) are enzymes with demonstratable activity in these cells. UDP-glucuronic acid transferase activity has been detected in C3H10T½CL8 cells by the formation of B(a)P-glucuronide conjugates (data not shown).

Various agents that have been shown to affect microsomal mixed-function oxidase activity *in vivo* were examined in this study in the C3H10T½CL8 transformation bioassay in an attempt to correlate the transformation results with specific levels of B(a)P metabolites formed.

Pretreatment of C3H10T½CL8 cells with benz(*a*)anthracene produced a similar increase in B(a)P-mediated transformation and total B(a)P metabolite formation. These transformation results are in close agreement with similar experiments performed in hamster embryo and mouse prostate systems (8, 16). Nesnow and Heidelberger (20) reported a 2-fold increase in 3-methylcholanthrene-mediated transformation in C3H10T½CL8 cells after a 48-hr pretreatment with benz(*a*)anthracene. The reduction of transformation elicited by benz(*a*)anthracene and the other modifiers at high concentrations could be due to a combination of effects such as toxicity and competition of modifier and B(a)P for metabolizing enzymes.

Pregnenolone-16α-carbonitrile also produced an increase in B(a)P-mediated transformation with an accompanying increase in B(a)P-7,8-diol formation. Pregnenolone-16α-carbonitrile is an inducer of hepatic cytochrome P-450 and aryl hydrocarbon hydroxylase in mice (11).

In the C3H10T½CL8 cells, 5,6-benzoflavone caused an increase in B(a)P-mediated transformation with a concomitant increase in total B(a)P metabolite formation. 5,6-Benzoflavone in the diet increased aryl hydrocarbon hydroxylase activity in A/HeJ mouse lung; however, it also inhibited the formation of B(a)P-induced pulmonary adenomas (29). The increased enzymatic capability induced by 5,6-benzoflavone *in vivo* seemed to cause an overall increase in detoxification of B(a)P, resulting in reduced tumorigenesis. 5,6-Benzoflavone produced results *in vitro* that differ from those reported *in vivo*. This might be due to differences between the responses of whole-animal

le 2

by C3H10T1/2CL8 cells

that which gave maximal increases in transformation: benz(*a*)anthracene, 1.0 μM; 5,6-benzoflavone, 10 μM; phenobarbital, 30 μM; pregnenolone-16α-carbonitrile, 10 were lysed, and B(a)P metabolites were extracted with ethyl acetate:acetone (2:1) and assayed by high-pressure liquid chromatography (27).

metabolite formed/hr/10^8 cells

1,6-Quinone	3,6-Quinone	6,12-Quinone	9-Phenol	3-Phenol	Total
0	13.7 ± 4.5 (6.5)	0	4.7 ± 1.3 (2.2)	14.8 ± 7.5 (6.8)	210.1 ± 56.3 (100)
1.0 ± 1.3 (0.3)	35.7 ± 17.2 (9.2)	0	12.9 ± 1.7 (3.3)	19.4 ± 0.4 (5.0)	388 ± 29.7 (100)
0.7 ± 0.5 (0.1)	2.1 ± 0.7 (0.6)	0	7.2 ± 3.9 (1.4)	14.4 ± 0.4 (4.8)	314.1 ± 12.0 (100)
0	3.0 ± 1.2 (0.3)	1.2 ± 0.6 (0.1)	10.5 ± 1.9 (1.8)	39.2 ± 3.4 (6.4)	636.8 ± 29.7 (100)
4.9 ± 3.4 (1.5)	36.9 ± 12.2 (9.4)	0.5 ± 0.7 (0.2)	5.7 ± 0.5 (1.3)	35.3 ± 15.1 (9.5)	408.4 ± 105.5 (100)
11.8 ± 2.6 (1.4)	85.6 ± 29.5 (9)	2.2 ± 2.8 (0.03)	10.0 ± 1.5 (1.6)	34.1 ± 5.7 (4.2)	702.4 ± 101.1 (100)
2.6 ± 0.4 (1.4)	15.4 ± 0.3 (7.8)	0.4 ± 0.2 (0.3)	4.8 ± 0.5 (2.2)	18.7 ± 2.0 (8.7)	204.4 ± 14.7 (100)
3.5 ± 0.5 (1.1)	28.5 ± 1.8 (9.9)	1.0 ± 0.2 (0.3)	5.4 ± 1.8 (2.4)	17.0 ± 0.2 (6.2)	283.7 ± 17.0 (100)
1.57 ± 0.38 (0.4)	6.4 ± 1.5 (1.8)	0	9.5 ± 2.6 (2.6)	17.4 ± 4.2 (5.7)	300.8 ± 15.1 (100)
0.8 ± 0.2 (0.3)	5.3 ± 0.1 (2.3)	0	6.4 ± 1.4 (2.3)	12.9 ± 0.3 (5.6)	246.5 ± 15.4 (100)

Table 3

Effect of agents on growth of C3H10T/12CL8 cells

Cells were seeded at 5000/dish; 4 days later, they were treated with the test agent which remained until cell harvest. The concentration of the agent was that which gave maximal transformation response. The cells were counted each day during log growth phase. The cell number was 2.6 ± 0.5 × 10^4 4 days after seeding.

Agent[a]	Cell no. (× 10^4)		
	5 days after seeding	6 days after seeding	7 days after seeding
Acetone (0.5%)	5.4 ± 2.4[b]	21.1 ± 2.3	49.8 ± 6.3
Benz(*a*)anthracene (1 μM)	8.6 ± 0.5	11.3 ± 5.8	27.2 ± 5.6
5,6-Benzoflavone (10 μM)	7.4 ± 2.0	8.9 ± 3.7	25.6 ± 5.7
Aroclor 1254 (3 μM)	8.3 ± 2.1	9.3 ± 3.2	14.7 ± 2.0

[a] Phenobarbital (30 μM) and pregnenolone-16α-carbonitrile (3 μM) gave values similar to those reported with acetone.
[b] Mean ± S.D. for 5 replicate plates.

systems and cell systems to environmental stimuli. Whole animals consist of different cell, tissue, and organ types while hamster embryo, mouse prostate, and C3H10T½CL8 cell systems consist of one major cell type, usually fibroblastic. The pharmacodynamic processes, which mediate the removal and excretion of a carcinogen and/or cocarcinogen and its metabolites and are operative in the whole animal, do not play a role *in vitro*. However, Kouri *et al.* (14) have reported that 2,3,7,8-tetrachlorodibenzo-*p*-dioxin increased both aryl hydrocarbon hydroxylase activities and tumorigenesis due to 3-methylcholanthrene in skin and s.c. tissues of mice, suggesting a cocarcinogenic effect.

Of the 5 agents tested, Aroclor 1254 produced the smallest increase in transformation in this cell system. B(a)P metabolite formation was also not affected by Aroclor 1254 treatment. The inability of Aroclor 1254 to induce mixed-function oxidase activity in C3H10T½CL8 cells is not understood. However, it has been reported that Aroclor 1254 inhibits 7,12-dimethylbenz(*a*)anthracene-induced tumorigenesis in mouse skin (7).

An increase in cell division stimulated by chemical treatment will result in an increased number of cells at risk at the time of B(a)P administration. The result of this increase in cell number should be an increase in the number of foci observed per dish. It has been demonstrated that this is not the case for all agents examined.

The results presented here indicate that agents which affect the metabolic pathways of chemical carcinogens will alter their effects on cell transformation. The stimulatory effect of benz(*a*)anthracene, 5,6-benzoflavone, phenobarbital, and pregnenolone-16α-carbonitrile on B(a)P-mediated cell transformation warrant their classification as cocarcinogens.

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